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Simultaneous determination of drugs and pesticides in postmortem blood using dispersive solid-phase extraction and large volume injection-programmed temperature vaporization-gas chromatography–mass spectrometry

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ABSTRACT

A d-SPE protocol followed by gas chromatography–mass spectrometry (GC–MS) analysis using large volume injection–programmed temperature vaporization (LVI–PTV) was optimized for simultaneous quantification of 14 pesticides, drugs of abuse, prescription drugs and metabolites in human postmortem blood without derivatization. The validated method showed good repeatability, linearity, intermediate precision, and recovery. LOQs were 0.02 or 0.03 μg/mL. The method showed to be fast and easy-to-implement in a forensic laboratory and was satisfactorily applied for the analysis of 10 postmortem blood real samples. Six samples contained cocaine (0.04–3.13 μg/mL), two 3,4-methylenedioxymethamphetamine hydrochloride (MDMA, 0.04–0.09 μg/mL) and two carbamazepine (0.08–0.98 μg/mL). Other analytes found were carbofuran (27.3 μg/mL), the metabolite 7-aminoflunitrazepam (1.12 μg/mL), amitriptyline (0.21 μg/mL) and diazepam (0.03 μg/mL).

1. Introduction

Pesticides, prescription drugs, and drugs of abuse are the major causes of fatal poisonings in the world, with pesticides mostly involved in suicides and drugs in accidental poisonings [1–8]. A recent study showed phenobarbital, diazepam, amitriptyline, cocaine and organophosphorus pesticides among the main chemicals involved in fatal poisoning in the Federal District of Brazil [8]. Chemicals can be detected in postmortem biological samples, including gastrointestinal tract material [8–10], intra-osseous fluid [11], larvae [12] and blood [13,14].

Detecting chemicals involved in fatal cases without an initial suspicion is an analytical challenge for forensic toxicologists. Immunoassay techniques are interesting for sample screening, although they present important limitations, including cross reaction between structurally similar molecules and the possibility of false negative/positive results, which require a confirmatory method [15]. Chromatographic methods are the best option for toxicological analysis, as they provide both the identification and quantification of the analytes [16–19].

An analytical method in forensic toxicology demands a protocol that guarantees the extraction of a wide range of substances with different physicochemical properties. Among the extraction techniques used for biological fluids, liquid–liquid (LLE) and solid phase (SPE) extractions are routinely used [19]. As disadvantages, LLE may require large volumes of solvents and often shows low efficiency due to matrix interference; SPE is time consuming and has a high cost, in addition to difficulties in extraction of compounds with different physicochemical properties [10,20]. Dispersive solid phase extraction (d-SPE) is a technique that requires a lower amount of sorbents and solvents; it does not need cartridges and column conditioning, among other advantages. d-SPE has been applied for the analysis of a wide range of compounds, including pesticides and drugs in different matrices [10,16,18,19,21–24]. Another technique that has been applied in forensic analysis is solid-liquid extraction with low temperature partitioning (SLE-LTP), including extraction of toxicants in postmortem samples [12,25]. It is a simple and efficient extraction/cleanup process, where the aqueous phase is frozen at low temperature, removing the soluble interferents, and the substances of interest remain in the organic phase.

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Gas chromatography coupled with mass spectrometry (GC–MS) is a simple and robust technique, and although it may present low sensitivity for some chemicals, the instrumentation is available in most forensic laboratories around the world [10,22–24,26–30]. An option to increase the sensitivity in GC analysis is a programmed temperature vaporizing injector for large volume injection (PTV–LVI). In addition to higher sensitivity, other advantages of the PTV–LVI technique are better recoveries of labile compounds and reduction of sample size, an important issue in the forensic area, due to the sample size limitation [31–33].

The aim of this study was the development and validation of a method for the determination of pesticides and prescription and abuse drugs in postmortem blood using PTV-LVI-GC–MS. Different extraction/cleanup protocols were tested during method development, and blood samples from real cases were analyzed using the validated method.

2. Materials and methods

2.1. Chemicals and reagents

Standards of haloperidol, diazepam, carbamazepine, bromazepam, phenobarbital and amitriptyline hydrochloride were kindly donated by the Brazilian Pharmacopeia (Rio de Janeiro, Brazil). 3,4-Methylenedioxymethamphetamine hydrochloride (MDMA), cocaine hydrochloride and 7-aminoflunitrazepam (7-AF) were provided by the United Nations Office on Drugs and Crime (UNODC). Carbofuran, terbufos, carbaryl, methiocarb and pirimicarb were purchased from AccuStandard (USA). Standard solutions of 1 mg/mL diazepam-d_5 and cocaine-d_3 (internal standards, IS) were purchased from Cerilliant – Sigma Aldrich (USA). Acetonitrile (ACN) LC–MS grade was obtained from Scharlau (Spain) and ethyl acetate (EtOAc) LC–MS grade was purchased from Merck (Darmstadt, Germany). Supelclean PSA (primary and secondary amine), magnesium sulfate anhydrous ($MgSO_4$) and sodium acetate (NaOAc) were purchased from Sigma Aldrich (USA). Ultrapure water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

Standards, stock solutions and working solutions were prepared in ACN. Stock solutions of 1000 μg/mL of each standard were used to prepare two mixed working solutions containing all analytes at concentrations of 10 μg/mL and 1 μg/mL. Working solutions of diazepam-d_5 and cocaine-d_3 at 1 μg/mL were used as internal standards (IS). All solutions were kept in amber vials at −20 °C.

Postmortem blood samples (no preservatives) were provided by the Forensic Medical Institute of the Federal District of Brazil (IML/DF). Control samples used during method development and validation gave negative results for all substances of interest in this study. Postmortem blood was collected from the femoral vein or cardiac cavity during necropsy and stored at −50 °C until analyzed.

2.2. Extraction method

Two extraction protocols were tested, d-SPE [19,24] and SLE-LTP [12], with modifications. In both methods, ACN and EtOAc were tested as extraction solvent. In the first step, common to both protocols, 1 mL of control postmortem blood was added to a 15 mL falcon-type tube and the sample spiked with a mixed working solution containing the 14 analytes of interest, each at 500 ng/mL, and the IS, each at 50 ng/mL. Two glass beads (2 mm) were added, the tube vortexed and 2 mL ACN or EtOAc added, vortexed; then 500 mg of a mixture of anhydrous $MgSO_4$:NaOAc (4:1) added, vortexed and centrifuged (3500 RPM/5 min). The supernatant was transferred to a 2 mL microtube.

In the d-SPE protocol, 50 mg of PSA and 150 mg of anhydrous $MgSO_4$ were added to the tube containing 1 mL of the supernatant, the tube vortexed and centrifuged (3500 RPM/5 min). In the SLE-LTP procedure, 500 μL of ultrapure water was added to the tube containing 1 mL of the supernatant, vortexed, centrifuged and left in the freezer overnight. In both protocols, the extract was transferred to a vial and analyzed by GC–MS.

Extraction recovery was determined by comparing the normalized mean area of control samples containing standards added pre-extraction with the normalized mean area of samples containing the standards added post-extraction, and expressed in %. All tests were performed in triplicate. Recovery rates between 80 and 120% and relative standard deviation (RSD) lower than 20% were the acceptance criteria for the method performance [34].

2.3. PTV-LVI-GC–MS conditions

Analyses were performed on an Agilent 7890A gas chromatograph equipped with a programmed temperature vaporizing injector for large volume injection (PTV–LVI), Agilent Multimode (MMI), coupled to an Agilent Technologies 5975C mass spectrometer Triple Axis detector (USA). A capillary column, DB-1 ms (30 m × 0.25 mm I.D., 0.25 μm film thickness, Agilent) was used. Helium was used as carrier gas. The oven temperature programing was initiated at 70 °C, with hold time 0 min, 20 °C/min to 200 °C held for 0 min, rate of 10 °C/min to 300 °C, hold time 4 min. The total run time was 20.5 min and solvent delay was 5.1 min. MS conditions were as follows: electron ionization mode; ionization energy 70 eV; ion source, 230 °C, interface heated to 280 °C.

The injector was set in the solvent vent mode and injection volume was 25 μL. Optimum conditions of the injector were evaluated by a series of injections of mixed standard solutions at 0.1 μg/mL, alternating the values of each parameter (initial temperature, initial time, rate, final temperature, vent flow, vent pressure, vent time, purge flow, purge time and injection speed) and evaluating the influence on the chromatographic peaks. Increasing the vent flow increases the solvent evaporation, but also leads to loss of the analyte. Higher vent pressure increases the solvent volume that reaches the column before the analytes are transferred, and lower vent time reduces the volume, but analytes can be lost when longer times are used. Initial temperature of injector should not be equal to or greater than the boiling temperature of the solvent. Optimum conditions of the injector were established as follows: initial injector temperature was set at 75 °C and held for 0.35 min, increased to 300 °C, hold time 0 min at a rate of 700 °C/min, followed by cooling post-injection to 200 °C, at a rate of 10 °C/min, vent flow (100 mL/min), vent pressure (3.5 psi), vent time (0.3 min), purge flow (50 mL/min), purge time (1.5 min) and injection speed (60 μL/min).

Chromatographic run was locked by Retention Time Locking – RTL method (Agilent), using the retention time of cocaine as reference (11.70 min). The system was controlled by the Agilent Chemstation GC–MS Software Version E 02.02.1431, and the result analyses by Agilent MassHunter Quantitative Analysis® software, version B 07.01, with a previously set method. The analytical method was performed in full scan (m/z 50–450) and in selected ion monitoring (SIM) modes. SIM mode analysis was performed by monitoring three ions for each analyte, except carbaryl and amitriptyline, for which two ions were used due to poor fragmentation in the mass spectrometer. For each analyte, including the IS, the ion of greatest abundance was chosen for quantification and the others as qualifiers. Table 1 shows the analyte retention times and the abundance of the fragments.

Table 1
Chemical structure, retention times (Tr), ions and fragment abundances for the 16 analytes and internal standards (IS).

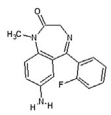
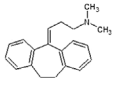
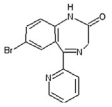
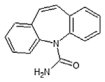
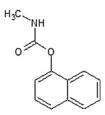
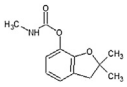
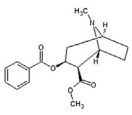
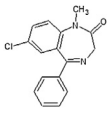
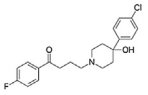
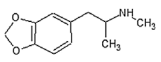
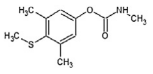
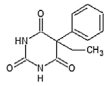
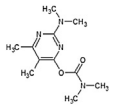
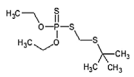
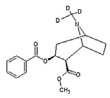
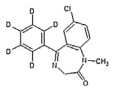
Substance	Structure	Tr	Ions[a] (m/z)	Abundance[b] (%)	IS
7-Aminoflunitrazepam		14.86 ± 0.03	283 255 264	100 ± 0.0 61.7 ± 1.3 14.1 ± 1.4	Diazepam-d_5
Amitriptyline		11.76 ± 0.01	58 202	100 ± 0.0 7.1 ± 0.31	Cocaine-d_3
Bromazepam[c]		14.80 ± 0.05	236 288 315	100 ± 0.0 15.1 ± 19.0 15.6 ± 18.3	Diazepam-d_5
Carbamazepine		9.83 ± 0.01	193 191 165	100 ± 0.0 15.2 ± 0.32 17.4 ± 0.34	Diazepam-d_5
Carbaryl		6.64 ± 0.00	144 115	100 ± 0.0 98.0 ± 11.9	Diazepam-d_5
Carbofuran		5.35 ± 0.00	164 149 131	100 ± 0.0 34.6 ± 0.84 84.0 ± 1.16	Cocaine-d_3
Cocaine		11.70 ± 0.01	182 82 303	100 ± 0.0 89.5 ± 2.5 23.3 ± 0.60	Cocaine-d_3
Diazepam		13.5 ± 0.01	256 283 221	100 ± 0.00 89.05 ± 0.92 28.35 ± 1.71	Cocaine-d_3
Haloperidol		17.22 ± 0.01	224 237 226	100 ± 0.0 80.0 ± 1.2 33.0 ± 0.60	Diazepam-d_5
MDMA		6.84 ± 0.01	58 77 135	100 ± 0.0 9.9 ± 3.54 9.4 ± 0.55	Diazepam-d_5
Methiocarb		6.88 ± 0.00	168 153 109	100 ± 0.0 63.6 ± 0.49 48.1 ± 0.94	Diazepam-d_5

Table 1 (*Continued*)

Substance	Structure	Tr	Ions[a] (m/z)	Abundance[b] (%)	IS
Phenobarbital[c]		9.77 ± 0.04	204 117 232	100 ± 0.0 32.2 ± 15.4 10.8 ± 5.3	Cocaine-d_3
Pirimicarb		8.77 ± 0.01	72 166 238	100 ± 0.0 146.6 ± 2.7 36.4 ± 2.1	Cocaine-d_3
Terbufos		8.41 ± 0.00	231 57 97	100 ± 0.0 109.1 ± 10.4 36.2 ± 3.1	Cocaine-d_3
Cocaine-d_3		11.69 ± 0.01	185 307	100 ± 0.0 4.5 ± 0.11	–
Diazepam-d_5		13.48 ± 0.01	262 290	100 ± 0.0 31.5 ± 1.43	–

MDMA: 3,4-methylenedioxymethamphetamine hydrochloride.
[a] The underlined ions were used for the quantification and the others as qualifiers.
[b] Expressed in mean ± standard deviation.
[c] Semi-quantitative analysis.

2.4. Method validation

The method was validated for linearity, limit of quantification (LOQ), carryover, selectivity, matrix effect, recovery, repeatability (intra-assay precision), intermediate precision and sample stability [34].

Linearity of the in-matrix standard curve was evaluated using seven different concentrations (0.02, 0.03, 0.08, 0.80, 1.6, 2.8 and 4.0 μg/mL) in triplicate, and post-extraction fortification with mixed working solution standards. The mean of normalized areas (analyte area/IS area) for each point was used for curve calculation, and a post-hoc test (Grubbs test) was performed to detect outliers. Homoscedasticity of the standard curve was evaluated for each analyte by Bartlett's test, and the curve was considered homoscedastic when standard deviations were not significantly different between the tested levels [35]. Parametric tests assume that data are homoscedastic (have the same standard deviation in different groups), so homoscedasticity should be evaluated [35]. Both tests were performed using the GraphPad Prism® software, version 6.01.

The carryover was evaluated by analyzing runs of a pool of five different postmortem blood samples, without addition of standards, after analysis of the highest concentration of the analytical curve, 4.0 μg/mL (blank, n = 3). The acceptance criterion was that the mean areas of the ion at the analyte retention time should not exceed 20% of the ion area signal at the lowest curve point. Selectivity was evaluated by analyzing 10 different blank matrix samples, without internal standard, to evaluate the presence of interferents at the analyte retention times.

Matrix effect and recovery were evaluated at the LOQ (low), 0.8 μg/mL (medium) and 4.0 μg/mL (high) concentrations. Three different sets of samples were used (n = 3 for each): analytical standards in solvent (a), standards added to a control matrix pre-extraction (b) and standards added to a control matrix post-extraction (c). Matrix effect was evaluated by comparing the sample normalized mean area obtained in samples with standards added post-extraction by the normalized mean area in samples with standards in solution, and expressed in %. Matrix effect was significant when change in the analyte response (suppression or enhancement) is higher than 20%. Recovery was calculated by comparing normalized mean area of samples with standards added pre-extraction with the normalized mean area of samples with standard added post-extraction, and expressed in %.

Repeatability and intermediate precision were evaluated using the control samples fortified at three different concentrations. For intermediate precision validation, the whole procedure was repeated on another day by the same analyst. LOQ of the method was defined for each analyte as the lowest level in which the method was validated (repeatability and intermediated precision, RSD < 20%; recovery in the range of 80–120%).

Stability of the extracted samples was evaluated under the laboratory conditions (25 ± 5 °C). Vials were placed on the GC–MS tray, and each sample analyzed in triplicate. Vials containing control samples fortified with standards (post-extraction) at 4.0 μg/mL were reanalyzed after 24 h, and control samples fortified at 0.08 and 0.80 μg/mL were reanalyzed after 48 h. Change in the concentration after the storage period should not exceed 20% for

the analyte to be considered stable under the laboratory conditions.

3. Results and discussion

3.1. Optimization of extraction

Fig. 1 shows the SIM of the 14 substances analyzed and the full scan chromatograms of a control sample using the four extraction protocols tested: d-SPE/ACN (E1); d-SPE/EtOAc(E2); SLE-LTP/ACN solvent (E3); SLE-LTP/EtOAc (E4). The E1 protocol shows a chromatogram with less interference from fatty compounds, such as lipids and cholesterol (identified by the mass spectral libraries), when compared to the other protocols (Fig. 1). The presence of fatty compounds was more evident (mainly at 18.5 min Tr) when the extraction solvent was EtOAc (E2 and E4), which is explained by its lower polarity compared to ACN (Fig. 1).

Fig. 2 shows the % recovery of the analytes from control matrix samples fortified at 0.5 μg/mL. Recoveries were out of the acceptable range (80–120%, ± 20% bias [34]) for 10 of the 14 analytes investigated in the E4 protocol, for seven analytes in the E3 protocol, for five in the E2 protocol and for two analytes in the E1 protocol (bromazepan and phenobarbital). The standard deviations were less than 20% for all compounds on protocol E1 only (data not shown), which also performed better in the recovery test for most compounds, and was selected for the method validation.

3.2. Method validation of the PTV-LVI-GC–MS analysis after d-SPE/ACN extraction

Linearity of matrix standard curve was calculated by the least squares method and showed to be satisfactory for most compounds ($r^2 \geq 0.99$), except for bromazepam and phenobarbital ($r^2 = 0.93$). No extreme values were observed (Grubbs test), and all curves showed to be homoscedastic (Bartlett's test).

Carry-over results were within the proposed acceptance limits for this parameter. No interfering peaks were observed in the SIM chromatogram of a control matrix, indicating that the method is selective.

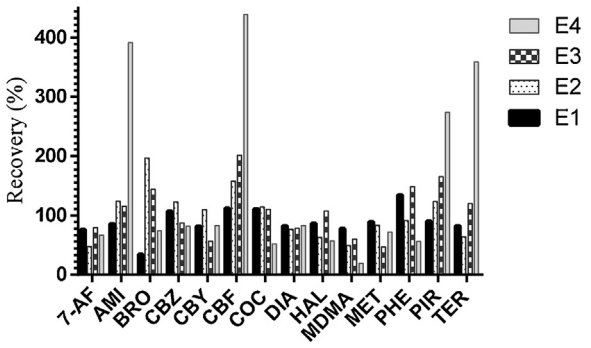


Fig. 2. Percent recovery of 14 analytes from control postmortem blood samples fortified at 500 ng/mL for the four clean-up/extraction protocols: E1: d-SPE with ACN; (E2): d-SPE with EtOAc; (E3): SLE-LTP with ACN; (E4): SLE-LTP with EtOAc. 7-AF: 7-aminoflunitrazepam; AMI: amitriptyline; BRO: bromazepam; CBZ: carbamazepine; CBY: carbaryl; CBF: carbofuran. COC: cocaine; DIA: diazepam; HAL: haloperidol: MDMA: 3,4-methylenedioxymethamphetamine hydrochloride; MET: methiocarb; PHE: phenobarbital; PIR: pirimicarb; TER: terbufos.

Matrix effects and validation parameters are shown in Table 2. In most cases, no significant effects (>20%) were observed at the two highest fortification levels tested (0.80 and 4.0 μg/mL). At the lowest level (0.02, 0.03 or 0.80 μg/mL), suppression of the signal was observed for 7-aminoflunitrazepan (7-AF), amitriptyline, bromazepam, carbamazepine and MDMA (up to 46%), and significant enhancement was observed for carbaryl, carbofuran and cocaine (from 50 to 131%). Signal enhancement due to the present of matrix is common in GC–MS analysis, and occurs due to the blockage of the active sites of injector liner, such as silane groups, by the matrix components, avoiding adsorption or thermal degradation of analytes [36]. Higher matrix effect at lower analyte concentration in a GC–MS was also reported by Schenck and Lehotay [37] and Godula et al. [38] for organophosphates and carbamates in food matrices. Wozniak et al. [39] reported signal enhancement for amphetamine, but ion suppression for phentermine in biological fluid, and Magalhães et al. [25] reported a significant matrix effect for cocaine determination in human liver, but it was not stated if it was enhancement or ion suppression.

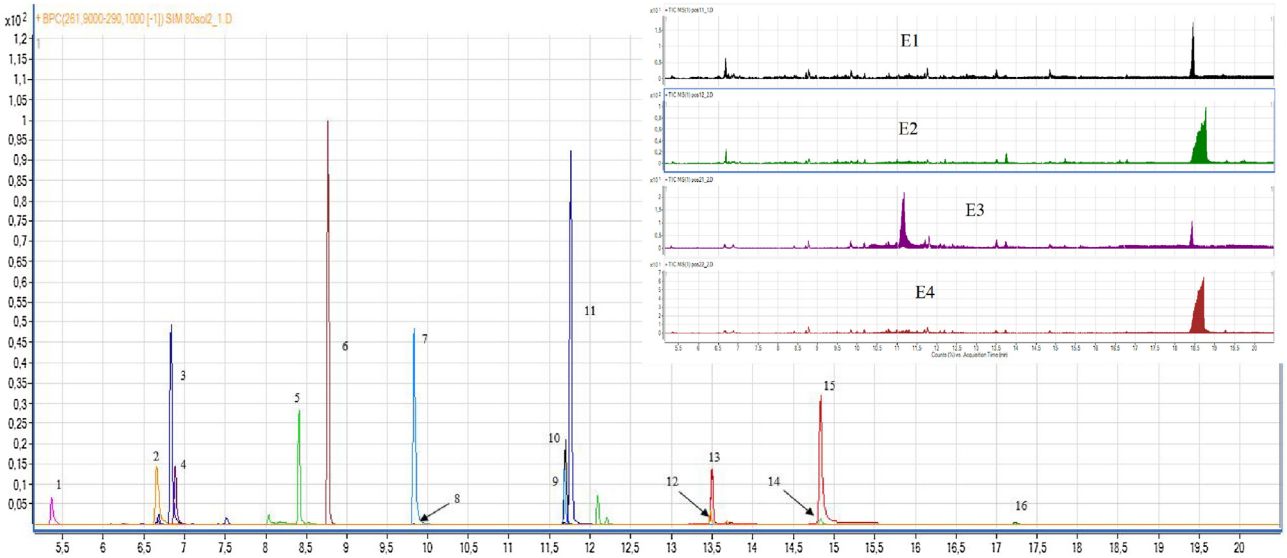


Fig. 1. Selected ion monitoring (SIM) of the 14 substances analyzed and internal standards in fortified control postmortem blood samples. (1) Carbofuran; (2) carbaryl; (3) MDMA; (4) methiocarb; (5) terbufos; (6) pirimicarb; (7) carbamazepine; (8) phenobarbital; (9) cocaine-d_3; (10) cocaine; (11) amitriptyline; (12) diazepam-d_5; (13) diazepam; (14) bromazepam; (15) 7-AF; (16) haloperidol. The insert shows the chromatograms (scan mode) of extracts from the four clean-up/extraction protocols tested. E1: d-SPE with ACN; (E2): d-SPE with EtOAc-solvent; (E3): SLE-LTP with ACN; (E4): SLE-LTP with EtOAc.

Table 2
Recovery, matrix effect, repeatability and intermediate precision (n = 3) of the 16 analytes in postmortem blood fortified at three concentration levels.

Substances	Concentration[a] (μg/mL)	Matrix effect (%)	Recovery (%)	Repeatability RSD (%)	Intermediate Precision RSD (%)
7-Aminoflunitrazepam	0.02	−39.5	106.8	17.0	17.4
	0.80	−19.8	63.8	4.8	4.9
	4.0	−22.6	72.7	5.1	6.9
Amitriptyline	0.02	−46.1	108.1	15.7	17.3
	0.80	−9.8	110.5	4.09	19.4
	4.0	−17.5	139.7	1.92	19.8
Bromazepam[b]	0.08	−37.7	25.8	14.0	19.4
	0.80	−29.3	34.7	8.0	15.9
	4.0	5.5	30.2	8.6	12.2
Carbamazepine	0.02	−23.7	74.6	3.5	12.2
	0.80	−13.9	101.3	3.4	19.8
	4.0	−21.5	111.3	5.7	17.4
Carbaryl	0.03	130.9	118.4	15.7	14.5
	0.80	2.0	100.6	4.2	4.2
	4.0	1.0	109.9	7.7	12.4
Carbofuran	0.02	122.6	93.3	9.0	11.7
	0.80	1.7	143.5	5.7	4.1
	4.0	4.7	142.3	5.4	10.5
Cocaine	0.02	52.5	116.3	4.45	11.4
	0.80	−8.12	108.3	3.94	1.82
	4.0	−2.5	103.2	0.94	3.64
Diazepam	0.02	−16.0	91.0	3.7	7.6
	0.80	−10.2	87.2	4.1	1.9
	4.0	−16.1	85.1	2.4	4.1
Haloperidol	0.03	9.1	115.8	9.1	17.89
	0.80	7.7	69.8	6.1	10.93
	4.0	1.47	68.4	5.2	5.3
MDMA	0.03	−35.8	86.7	5.3	19.73
	0.80	−10.8	100.0	6.9	14.52
	4.0	−35.6	104.6	7.2	15.59
Methiocarb	0.03	5.4	116.4	12.35	16.96
	0.80	−0.55	99.2	4.0	6.94
	4.0	−0.31	101.8	4.36	9.21
Phenobarbital[b]	0.08	NA	99.5	15.7	16.65
	0.80	−50.5	55.9	11.0	18.87
	4.0	9.1	21.3	17.4	19.11
Pirimicarb	0.03	−3.2	107.3	17.1	19.44
	0.80	−15.3	119.8	2.6	9.83
	4.0	−12.5	118.3	4.7	16.08
Terbufos	0.02	−0.17	87.5	11.6	16.42
	0.80	−30.9	102.7	9.0	15.76
	4.0	−16.5	108.1	8.4	13

MDMA: 3,4-methylenedioxymethamphetamine hydrochloride; RSD: relative standard deviation; NA: data not available.
[a] Underlined concentrations are the LOQ.
[b] Semi-quantitative analysis.

For most analytes, recoveries were within the acceptable range at all concentrations (80–120%), and repeatability and intermediated precision were below 20% in all cases (Table 2). Bromazepan showed poor recovery at all fortification levels (<50%), and phenobarbital showed recovery <60% at the two highest levels.

As many real samples may contain the analytes at low concentration, in-matrix standard curves were used in this study to determine the concentration in the postmortem blood samples. Validation results for bromazepam and phenobarbital showed inadequate linearity ($r^2 < 0.93$), and recoveries were enough only for a semi-quantitative analysis. For the other analytes, the LOQ was set at the lowest concentration tested (0.02 or 0.03 μg/mL; Table 2). The stability study showed that all analytes were stable when left on the instrument trail for up to 48 h, with at least 80% of the analyte remaining after the period (Fig. 3).

After death, the blood undergoes numerous changes due to the decomposition process [13,14,40]. In many cases, samples collected during autopsy are denatured by putrefaction, with clots and hemolysis [19], so a specific method for this biological matrix needs to be performed [19]. A few studies report the use of d-SPE followed by GC–MS analysis of postmortem human blood [22–24], but the protocols were not validated for this matrix. To the best of our knowledge, this is the first study that has performed a full validation of a d-SPE extraction GC–MS method for multiclass chemicals in postmortem blood. Furthermore, previous studies have reported validation for 13 analytes [22], eight

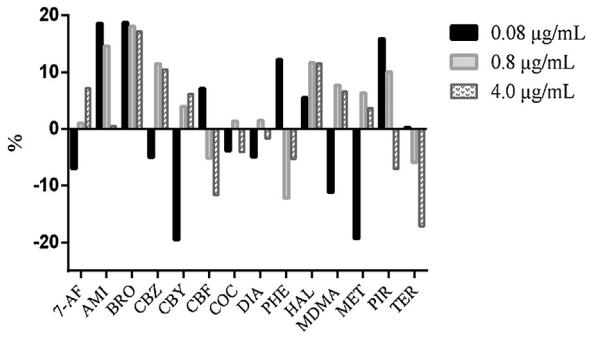


Fig. 3. Stability of the analytes in postmortem blood extracts under laboratory conditions. Results are mean % of the initial concentration (t = 0) after 48 h (for 0.08 and 0.80 μg/mL) and 24 h (for 4.0 μg/mL), n = 3 at each concentration. 7-AF: 7-aminoflunitrazepam; AMI: amitriptyline; BRO: bromazepam; CBZ: carbamazepine; CBY: carbaryl; CBF: carbofuran. COC: cocaine; DIA: diazepam; PHE: phenobarbital; HAL: haloperidol: MDMA: 3,4-methylenedioxymethamphetamine hydrochloride; MET: methiocarb; PIR: pirimicarb; TER: terbufos.

pharmaceuticals [24] or a semi-quantitative drug screening for 65 chemicals by using a d-SPE/GC–MS method [23], which demonstrates that the number of substances included in our study is in accordance with others reported. The use of a large volume of injection (LVI) allowed this study to reach similar LOQs to those demonstrated by using GC–MS ion-trap method [24].

The validated GC–MS is suitable for routine analysis, showing appropriatted LOQs for detecting toxic concentration of the tested substances, in addition to responding adequately at a wide range of concentrations, an important aspect in forensic science. An advantage of this method is that GC–MS is a technique that is easier to handle and more robust when compared to liquid chromatography, in addition to being more widely available in forensic laboratories. One disadvantage is that the method is not suitable for the analysis of polar compounds of forensic interest, such as aldicarb.

3.3. Real case samples

The validated method was used for the analysis of postmortem blood samples from real forensic cases, and the results are showed in Table 3. Samples that contained the analyte at a concentration higher than the standard curve range were diluted for the quantification. Cocaine was found in 60% of the samples, alone or in combination with 7-AF or carbamazepine. In case 2, the victim was found hanged at home, and a bluish fluid was found in her oral cavity and in the gastric content. The blood concentration of cocaine found in the postmortem blood (3.13 μg/mL) would be enough to cause death [41]. The sample also contained 1.12 μg/mL of the flunitrazepam metabolite, 7-AF, a level that could also be fatal. Hasegawa et al. [42] described a suicide case in which the victim could have ingested up to 60 tablets containing 2 mg of flunitrazepam each; 7-AF was found at 1.4 μg/mL in left heart blood and at 0.40 μg/mL femoral vein blood. Furthermore, Jones et al. [43] reported mean femoral blood levels of 7-AF of 0.40 μg/mL in 28 mono-intoxication cases of death. In case 10, the victim felt sick at home, was taken by paramedics, but died before arriving at the hospital. The cocaine blood concentration (1.22 μg/mL) suggests an overdose case. According to Schulz et al. [41] a cocaine blood/plasma concentration from 0.25 ng/mL upwards is enough to be toxic dose and from 0.9 μg/mL to 2.1 μg/mL it can lead to coma or death.

Table 3
Postmortem blood samples analyzed by the validated analytical method.

Case number	Sex/age	Substance	Concentration (μg/mL)
1	M/39	Cocaine	0.38
2	F/15	7-Aminoflunitrazepam	1.12
		Cocaine	3.13
3	M/51	Carbofuran	27.3
4	M/20	Carbamazepine	0.98
5	M/31	Cocaine	0.06
6	M/32	Cocaine	0.15
7	M/23	MDMA	0.04
8	M/31	Amtriptyline	0.21
		MDMA	0.09
		Diazepam	0.03
9	M/19	Carbamazepine	0.08
		Cocaine	0.04
10	M/38	Cocaine	1.22

MDMA: 3,4-methylenedioxymethamphetamine hydrochloride; M: male; F: female; age in years.

Carbofuran was the only pesticide detected in the samples (case 3), related to a man found dead at home. Gastric residual contents findings indicated death due to ingestion of “chumbinho”, an illegal rodenticide sold in Brazilian street markets that contains pesticides of the carbamate (mostly aldicarb and carbofuran) and organophosphorus classes (terbufos) [44], frequently involved in fatal intoxications [8]. The calculated concentration of carbofuran (27.3 μg/mL) is compatible with forensic fatal cases already reported [45–47].

One limitation of this study is that benzoylecgonine, the main cocaine metabolite, was not included in the validated method. Benzoylecgonine was tested in the four extraction protocols, but the results were not satisfactory for any of the parameters, similar to what was reported by Alves et al. [48] using d-SPE and GC–MS analysis. Considering that cocaine is rapidly metabolized (half-life of 0.7–1.5 h) [49,50], it would be interesting to include other metabolites in future work, such as ecgonine methyl ester. Flunitrazepam was also not included, although other studies showed that ingestion of flunitrazepam was confirmed by detection of the major metabolite, 7-AF, with no detection of the drug [43,51].

4. Conclusions

In this study, a modified d-SPE method followed by PTV-LVI-GC–MS analysis was validated for toxicological analysis in postmortem blood for detection and quantification of pesticides, prescription and illegal drugs. To the best of our knowledge, this is the first study to validate a d-SPE method with PTV-LVI-GC/MS for postmortem blood samples. The method is simple to execute, fast and of low cost; it was successfully applied for the analysis of real case samples and can be introduced for a routine analysis in a forensic laboratory.

Author’s contribution

Ettore Ferrari Junior: Conceptualization, Methodology, Writing-original draft preparation.

Eloisa Dutra Caldas: Supervision, Data curation, Writing-reviewing and editing.

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